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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :

YUTARO KANEKO ET AL. : EXAMINER: WILSON, M.

SERIAL NO: 09/087,513 :

FILED: MAY 28, 1998 : GROUP ART UNIT: 1632

FOR: METHOD FOR INDUCING IMMUNITY TO VIRUSES :

DECLARATION UNDER 37 C.F.R. § 1.132

COMMISSIONER FOR PATENTS
ALEXANDRIA, VIRGINIA 22313

SIR:

Now comes ALAGARSAMY SRINIVASAN who deposes and states that:

(1) I am a graduate of JAWAHARLAL NEHRU UNIVERSITY and received a Ph.D degree in the year 1977.

(2) I have been employed by THOMAS JEFFERSON UNIVERSITY for 9 years as a researcher in the field of AIDS PATHOGENESIS AND VACCINES.

(3) I understand the English language or, at least, that the contents of the Declaration were made clear to me prior to executing the same.

(4) I understand that the U.S. filing date of the above-identified application is May 29, 1998.

(5) I have read and am familiar with the contents of the above-identified application. Specific portions of the specification are discussed below.

(6) The field of the invention described in the above-identified application is methods and compositions for inducing immunity against the human immunodeficiency virus (HIV).

(7) Page 1, lines 5-10 of the specification of the above-identified application discusses the field of the invention and reads as follows:

Field of the Invention

> The present invention relates to methods for inducing immunity to viruses, and in particular to viruses which undergo mutation. More particularly, the invention relates to methods for increasing the response of cytotoxic T lymphocytes (CTL) against the viruses using a construct which expresses a mutant form of the envelope (env) glycoprotein of the virus containing an altered or deleted immunodominant epitope.

(8) Page 1, line 11 to page 3, line 18 of the specification of the above-identified application discusses the background of the invention described in that application. In particular, that section of the application V3 loop of the HIV protein gp120. See page 2, lines 13-16 and page 3, lines 3-6.

(9) Page 3, line 19 to page 5, line 9 of the specification of the above-identified application provides a summary of the invention described therein. In particular, page 3, line 20 to page 4, line 4 reads as follows:

SUMMARY OF THE INVENTION

> Accordingly, one object of the present invention is to provide a method for inducing cellular immunity to a virus by administering to a patient a nucleic acid encoding an envelope glycoprotein of the virus, in an amount sufficient to induce cellular immunity against the virus, wherein the envelope glycoprotein
>
> (a) contains a modified immunodominant epitope; and
>
> (b) induces cellular immunity to a conserved epitope of the envelope glycoprotein.

(10) Page 4, lines 12-23 of the specification of the above-identified application reads as follows:

> Another object of the invention is to provide a method for preparing a vaccine against a virus including:
>
> (a) introducing into a vector DNA or liposome a nucleic acid encoding an envelope glycoprotein of the virus, wherein the envelope glycoprotein contains a modified immunodominant envelope; and
>
> (b) mixing the vector DNA or liposome with a suitable adjuvant.
>
> Another object of the invention is to provide a method for preparing a vaccine against a virus including:
>
> (a) introducing into antigen presenting cells (APCs) a nucleic acid encoding an envelope glycoprotein of the virus, wherein the envelope glycoprotein contains a modified immunodominant envelope; and
>
> (b) mixing the APCs with a suitable adjuvant.

(11) Page 9, lines 20-21 of the above-identified application reads as follows:

> A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

(12) Page 22, line 14 to page 23, line 8 of the specification of the above-identified application reads as follows:

> The virus which contains the envelope glycoprotein is preferably a virus which undergoes antigenic variation such as by antigenic drift (e.g., lentiviruses) or antigenic shift (e.g., influenza). Preferred viruses are retroviruses, and particularly lentiviruses such as human immunodeficiency virus (HIV), equine infectious anemia virus (EIAV), caprine arthritis encephalitis virus (CAEV), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), and visna virus.
>
> As noted above, preferred immunodominant epitopes include the third variable loop (V3) of an envelope glycoprotein, a neutralization epitope, and a fusion epitope. However, it is noted that fusion epitopes may be highly hydrophobic, and thus not well exposed on the glycoprotein. In some cases, cleavage of a precursor glycoprotein, such as the gp160 glycoprotein of HIV into a gp120 and gp41 is required to expose such an epitope.

Any method of altering the immunodominant epitope may be used, including deletion of the epitope, insertion of sequences which disrupt the secondary and tertiary structure of the epitope, and modification by sequences by site-directed mutagenesis. It will be appreciated that alteration by conservative substitution of amino acids will be the least disruptive of the epitope, while substitution of amino acids of different size, structure or charge will have a more significant effect on the structure.

(13) Example 2 of the above-identified application at page 26, line 10 to page 27, line 14 describes the preparation recombinant viruses and reads as follows:

The HIV-1IIIB isolate was the source of the full-length env gene and the ΔV3 loop mutant cloned in the pSCII-based vector under the control of a synthetic early/late vv promoter (Earl et al, 1990, Removal of cryptic poxvirus transcription termination signals from the human immunodeficiency virus type 1 envelope gene enhances expression and immunogenicity of a recombinant vaccinia virus. *J Virol. 64:2448-2451*). The vv-ΔV3 mutant with the Δ297-329 deletion (15 incorporated by reference herein in its entirety) was constructed by ligation of fragments obtained by PCR amplification from the pSVIII-env plasmid (a gift from Dr. J. Sodroski, Dana-Farber Cancer Institute, Boston, MA). One fragment was generated by PCR with the synthetic oligonucleotide containing the *SalI* site and the CCACC Kozak's sequence in front of the ATG codon (5'-AGAGTCGACCCACCATGAGAGTGAAGGAGA-3', sense) (SEQ ID NO: 1), and the oligonucleotide (5'-ACAGGTACCCCA TAATAGACTGTGAC-3', antisense) (SEQ ID NO:2) containing the *KpnI* side, used for ligation with the second env fragment. The second fragment was derived by *KpnI* and *Bam*HI digests of the pSVIII-env plasmid, and the third fragment was generated by PCR with the synthetic oligonucleotide containing the *Bam*HI site at its 5' end (5'-AACGGATCCTTAGCACTTATCTGGG-3', sense) (SEQ ID NO:3) and the antisense primer (5' TTGCGCGGCCGCTTATAGCAAAATCCT TTCC-3') (SEQ ID NOA) containing the TAA stop codon followed by the *NotI* site. The three fragments were ligated into the *SalI* and *NotI* sites of the pSC11-based vector (a generous gift of Dr. L. Eisenlohr, Thomas Jefferson University, Philadelphia, PA) to generate plasmid pSC-ΔV3. A similar approach was used to generate plasmid with the WT env gene (pSC-WTP) using recombinant clone pIIIB (Hwang, et al., *Science* 253:71-74) kindly provided by Dr. B. Cullen (Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC). Plasmids pSC-ΔV3 and pSC-WTP were used to generate vv- ΔV3 and vv-WTP by homologous recombination as described (Earl et al, 1990, *J Virol. 64:2448-2451*).

(14) Example 14 of the above-identified application at page 34, line 17 to page 35, line 14 describes Expression of ΔV3 and WT env glycoprotein in vv-infected cells and reads as follows:

> To examine structure-function relationships between the HIV env glycoprotein and cellular responses to its mutated gene products, a set of recombinant vaccinia viruses was constructed with complete and V3 loop-deleted env genes. Immunoprecipitation of env gene products from metabolically labeled JA2 cells with serum of an HIV-infected individual revealed less than 20% variation among representative recombinant clones (Fig. 1). For functional studies, two clones designated vv-7ΔV3 and vv-WTP-2 were selected with comparable levels of expression of the full-length and mutated env glycoproteins, respectively.
>
> Immunofluorescence staining and immunoprecipitation of env glycoproteins from vv-infected cells (Fig. 2A and B) revealed that cells expressing the 7ΔV3 mutant did not bind the G3-523 anti-V3 loop mAb. They exhibited lower binding of F105 mAb directed to the CD4-binding site of gp 120, consistent with previous reports which demonstrated structural relationship between the V3 loop and the C4 region of gp120 glycoprotein (Wyatt, R., M. et al., *J. Virol. 66:6997-7004.*). Despite the differences in binding to the F105 mAb, the reactivity of the vv-7ΔV3-infected cells with serum of an HIV-infected individual suggests that the folding of the oligomeric gp120 on the surface of infected cells has been preserved in the V3 loop-deleted mutant.

(15) Example 15 of the above-identified application at page 35, line 17 to page 37, line 37 describes induction of env-specific CTL responses by the vv-7ΔV3 env mutant and reads as follows:

> The env-specific CTL activity was induced in PBMC. of HIV-infected individuals by stimulation with anti-CD3 mAb and autologous LCLs expressing either the wild-type or the V3 loop-deleted env glycoprotein. Because the patients were HLA-$A2^+$, JA2 cells infected either with vv-WTP-2 or the HIV- IIIIB isolate were used as targets in the ^{51}Cr-release assay. All HIV-infected individuals exhibited env-specific CTL activity in anti-CD3 mAb-induced cultures of PBMC with specific lysis that varied between 11% and 26% (E:T ratio of 10:1; Fig. 3, left panel). In 4 out of 6 patients, the stimulation of PBMC with autologous LCLs expressing the env glycoproteins resulted in substantial increases of the cytotoxic response. However, the degree of lytic activity varied in individual cultures. For example, in patients 417, 521, and 428 the CTL activity was higher in cultures induced with cells expressing the 7ΔV3 mutant, whereas in the remaining patients the responses to the full-length env gene products were either higher or comparable to those induced by the 7ΔV3 gene products, and similar to nonspecific responses induced

with anti-CD3 mAb. Similar variation of CTT, activity was detected against JA2 cells infected with the HIV- 1IIIB isolate using a limiting dilution assay. In 3 HIV-infected individuals, the frequencies of CTLp directed against HIV-1IIIB-infected JA2 cells ranged from 49 to 216 CTL/10^6 PBMC (Fig. 3, right panel). The highest numbers of CTLp were detected in patients 521 and 428 who also showed increased levels of CTL activities in the bulk cultures of induced PBMC.

The heterogeneity of the CTL responses in the induced cultures suggests that differences in the immunogenicity of the complete and mutated env gene products might influence the repertoire of expanded CTL clones in HIV-infected patients. To explore this issue further, CTL activity was tested against vv-7ΔV3- and vv-WTP-2-infected JA2 cells in cultures induced with synthetic peptides D1 (aa, 120-128), D2 (aa. 814-823) and I-10 (aa, 318-327), representing at least three distinct epitopes of the env glycoprotein. In patient 417, who was capable of eliciting CTL responses to all three peptides, the highest activity against the env was detected in cultures induced with peptide D1, whereas the responses to peptides I-10 and D2 were diminished (Fig. 4A). The molecular basis for the lower responses to peptides I-10 and D2 might be related to the increased divergence within autologous viral sequences in these epitopes exhibited by the prevailing HIV isolate of the patient (Fig. 4B). This is in agreement with previous reports demonstrating sequence variation within the V3 loop and the C-terminal region of env glycoprotein of primary HIV isolates (Hwang et al, 1991, *Science 253: 71-74.*, Dupuis, M., S. 1995, *J. Immunol. 155:2232-2239.*).

It is also notable that cultures induced with the dominant peptide D1 were more efficient in lysing JA2 cells expressing the 7ΔV3 mutant than those induced with the full-length env gene products, and were associated with the prevalence of $CD8^+$ T cells (i.e. >70% of total CD3+ T cells). The higher responses against the mutated env gene products in cultures induced with the D1 peptide is in agreement with the increased frequencies of D1-specific CTLp in PBMC of two HIV-individuals stimulated with vv-7ΔV3-infected LCLs as compared to those induced with cells expressing the WTP-2 env gene products (20 and 55 CTL/10^6 PBMC vs. 12 and 32 CTLp/10^6 PBMC, respectively). Results of these experiments suggest that deletion of the V3 region redirects the immune responses to conserved epitopes of the env glycoprotein in some HIV-infected individuals.

(16) Example 16 of the above-identified application at page 37, line 14 to page 38, line 8 describes ADCC against 7Δ V3 mutant-infected targets and reads as follows:

In a subsequent study, the susceptibility of cells expressing the 7ΔV3 mutant and WTP-2 env glycoproteins to ADCC-mediated lysis was studied using PBMC of HIV-seronegative donors and polyclonal Ig, purified from sera of HIV-infected individuals. Results of experiments with PBMC of 3 HIV-seron gative individuals revealed 15% - 20% of

specific lysis against vv-WTP-2-infected autologous LCLs at the E:T ratio of 50:1 (Fig.5). Among five mAbs specific for epitopes within the V2 and V3 loops (697-D and 694/98-D), the CD4-binding site (F105), the C-terminus (670D), and the gp41 region of gp160 (50-6911), the highest ADCC activities against the WTP-2-infected target were mediated by the 694/98-D and F 105 mAbs. Antibodies directed to other regions of gp 120 had less effect on the ADCC-mediated lysis. In contrast, the specific lysis detected against vv-7ΔV3-infected target cells in the presence of polyclonal anti-gp120 antibodies or the F105 mAb was less than 4% (Fig. 5). The increased resistance of the vv-7ΔV3-infected cells to the ADCC-mediated lysis might be related to conformational changes in the CD4-binding region induced by deletion of the V3 region, as well as lack of reactivity of this molecule with the anti-V3-specific antibodies present in the majority of HIV-infected individuals.

(17) Example 17 of the above-identified application at page 38, line 9 to page 42, line 14 describes syncytium formation and induction of apoptosis by the 7ΔV3 mutant and reads as follows:

The role of the V3 loop in the mechanism of induction of apoptosis was analyzed in $CD4^+$ SupT-1 cells infected with vaccinia viruses expressing the complete and mutated env glycoproteins. Examination of SupT-1 cells infected with vv-WTP-2 under a light microscope revealed cell clumping followed by appearance of syncytia 12 hr after infection, and routinely, 80% to 90% cells formed giant cells 24 hr after infection. No syncytia were observed in cultures infected with either vaccinia virus alone or the vv-7ΔV3 mutant.

The apoptotic process associated with syncytium formation was analyzed in individual cultures 16 hr after infection. Analysis of DNA content by staining of nuclei with PI indicated that infection of cells with the full-length env glycoprotein resulted in appearance of ~20% of cells with a sub-G1 DNA content representing apoptotic cells (Fig. 6A), concomitant with decreases in the proportion of cells in the G1 phase of the cell cycle (from 39% to 15%). In contrast, the numbers of apoptotic cells in uninfected cultures and cultures infected with vaccinia virus or the 7ΔV3 mutant were lower than 5%. Apoptosis in Sup-T1 cells expressing the wild-type env glycoproteins was also confirmed by visualization of nucleosome-sized DNA multimers of 180-200 by to form the characteristic "step-ladder" appearance after size separation on agarose gel (Fig. 6B). Sup-T1 cells infected with vac or expressing the 7ΔV3 mutant failed to induce DNA fragmentation over background levels in isolated low molecular weight DNA.

The induction of apoptosis during infection of SuPT-1 cells with vv-WTP-2 is in agreement with previous studies which demonstrated that full-length HIV env glycoproteins are responsible for syncytium formation and apoptosis (Corbeil et al, *J. Exp. Med. 183:39-48*; Laurent-Crawford et

al, 1993, *AIDS Res. and Human Retroviruses 9:761-773*). However, it has been shown that HIV only rarely induces apoptosis in infected cell *in vivo* (Finkel et al, 1995, *Nature Medicine 1:129-134*). To analyze the effect of a deletion of the V3 region on induction of apoptosis in uninfected cells, IL-2-dependent $CD4^+$ T lymphocytes were induced through the TCR/CD3 complex after incubation with the full-length and mutated env glycoproteins expressed on the surface of APC. For induction of apoptosis, vv-7ΔV3- and vv-WTP-2-infected THP-1 cells were fixed with paraformaldehyde and incubated with IL-2-dependent T lymphocytes for 2 hrs to allow contact between CD4 expressed on T cells and cell surface-associated gp120 prior to stimulation with anti-CD3 mAb. Induction of apoptosis, monitored after 72 hrs by staining of nuclei with PI and flow cytometry analysis, revealed less than 7% of fragmented DNA in cultures infected with vac and the vv-7ΔV3 mutant. On the other hand, T cells preincubated with the full-length env glycoprotein prior to activation, revealed DNA fragmentation approximately three times that of control levels (Fig. 6C).

(18) Page 40, line 1 to page 42, line 7 of the above-identified application reads as follows:

Although autologous LCLs expressing comparable levels of the full-length and mutated env glycoproteins were capable of inducing CTL activity in PBMC of HIV-infected individuals, cultures stimulated with the mutated env gene products exhibited increased activity against conserved epitopes of the env glycoprotein. Although not intending to be bound by theory, the mechanisms responsible for the increased immunogenicity of the conserved epitopes in the ΔV3 mutant might be related to lower responses of the HIV-infected individuals to V3 due to increased natural sequence variation within this region. Alternatively, induction of CTL responses with APC expressing the full-length env glycoprotein might be less efficient as compared to those stimulated with the mutated env gene products because binding of the entire gp120 to CD4 on uninfected $CD4^+$ T cells may suppress the function of these cells, thus reducing help needed for optimal induction of CTL activity (Giovarelli et al, 1988, *J. Immunol. 141:2831*; Landolfo et al, 1985, *Science 229:176*; Romagnani, 1991, *Immunol. Today 12:256*). In addition, it has been demonstrated that free I-10 peptide corresponding to the epitope located within the V3 loop of the HIV-111B isolate can inactivate murine $CD8^+$ CTL by a self-veto mechanism which involves simultaneous occupancy of the MHC class I molecule and the TCR on the same CTL (Takahashi et al, 1996, *J. Exp. Med. 183:879-889*). The possible implication of this phenomenon for HIV pathogenesis in humans is that when virus-infected cells are lysed and the digested intracellular proteins released into the environment of T cells, the V3 loop-related self-veto effect may inhibit the induction of CTL responses.

The findings that deletion of the V3 region resulted in ~80% reduction of ADCC might be related to the absence of the V3 loop which

serves as a target for anti-V3 loop-specific antibodies abundant in sera of HIV-infected individuals (Vogel et al, 1994, *J. Immunol. 153:1895-1904*). Secondly, some conformational changes in other epitopes of gp120 may also contribute to a lower binding of some anti-gp120 antibodies, including those directed to the C4 region of the env glycoprotein. The fact that deletion of the V3 loop resulted in lack of both syncytium formation and induction of apoptosis in $CD4^+$ cells is consistent with the notion that these processes are triggered by an interaction between cell membrane expression of mature env glycoproteins and the CD4 receptor (Corbeil et al, 1996, *J. Exp. Med. 183:39-48*; Tuosto et al, 1995, *Eur. J. Immunol. 25:2907-2916*). The recently discovered physical association between the CD4-gp120 complex with CXCR-4 and CCR-5 co-receptors on cell membranes (Trkola et al, 1996, *Nature 384:184-187*; Lapham et al, 1996, *Science 274:602-604*) suggests that these interactions may initiate events leading to induction of cell death in a particular subset of $CD4^+$ cells. It has been suggested that the V3 loop is involved in these interactions (Feng et al, 1996, *Science 272:872-877*), and that its structure may contain or influence the nature of a complex binding site for the chemokine receptors on gp120 (Trkola et al, 1996, *Nature 384:184-187*). For example, it has been shown that deletion of the V3 loop from both HXB2 gp120 and JR-FL gp120 destroys the CD4-induced epitopes for mAbs 48d and 17b (Wyatt et al, 1992, *J. Virol. 66:6997-7004*; Thali et al, 1991, *J. Virol. 65:6188-6193*), which may be relevant to the inability of the ΔV3JR-FL gp120 to interact with CCR-5 (Trkola et al, 1996, *Nature 384:184-187*), and single amino-acid changes in the V3 and C4 regions of HIV-1LAI also have major effects on the structure of these epitopes (Moore et al, 1993, *J. Virol. 67:4785-4796*).

Vaccination with the ΔV3 mutant induces immune responses to potentially more conserved regions of the env glycoprotein, which will also reduce the need for immunization with HIV strain-specific env glycoproteins. For induction of cellular responses, the ΔV3 mutant could be injected as a plasmid DNA (Okuda et al, 1995, *AIDS Res. Hum. Retrovir. 11:933-943*) or expressed in autologous cells by intracellular immunization (Wu et al, 1996, *J. Virol. 70:3290-7*) prior to injection into the host. In either case, the cells expressing the mutated env gene products are likely to escape from the ADCC-mediated lysis *in vivo* and may not interfere with functional activities of other $CD4^+$ cells. The fact that the ΔV3 mutant interacted with anti-gp120 antibodies of HIV-infected individuals suggests that it is capable of inducing antibody responses *in vivo*.

It should be also noted that although the data showed that the ΔV3 mutant does not induce apoptosis in cells in which it is expressed, it is currently unknown to what extent cells expressing the mutated env glycoprotein are susceptible to apoptosis induced by the full-length env glycoprotein.

(19) I have read and am familiar with the contents of Claims 14, 15, 19, and 21-36 of the above-identified application, a copy of which is attached hereto as Exhibit 1.

(20) I have read and am familiar with the contents of the Official Action dated June 25, 2003 in the above-identified application.

(21) The amino acid sequence of the envelope glycoprotein of HIV was well-known to those skilled in the field of the invention at the time the present application was filed in the U.S. In particular, the location of the hypervariable region of the amino acid sequence was well-known to those skilled in the field of the invention. In addition, it was well-known to those skilled in the field of the invention that the third variable loop (V3) of includes amino acids 297 to 329. Thus, those skilled in the field of the invention at the time the present application was filed in the U.S would have appreciated that a reference those numbers was referring to the amino acid sequence of the protein and not to the nucleic acid sequence encoding that protein. That this is so is demonstrated by Back et al., *Journal of Virology*, Nov. 1993, pp. 6897-6902, a copy of which is attached hereto as Exhibit 2. Back et al. describe the gp41 coding region and the hypervariable region. See Figure 2 at page 6900 of Back et al., which explicitly describes that amino acids 297 to 329 are part of V3, i.e., the third variable loop.

(22) Those skilled in the field of the invention at the time the present application was filed in the U.S would have appreciated that the vv-ΔV3 mutant with the Δ297-329 deletion prepared in Example 2 of the above-identified application was a construct in which amino acids 297-329 of V3 were replaced with Gly-Ala-Gly. That this is so is demonstrated by the facts that Example 2 of the above-identified application describes that the vv-ΔV3 mutant was constructed from ligation of fragments obtained by PCR amplification from the pSVIII-env plasmid, which was a gift from Dr. J. Sodroski. See page 26 of the above-identified application. The pSVIII-env plasmid is described in a scientific publication co-

authored by Dr. Sodroski: Wyatt et al., Journal of Virology, Dec. 1992, pp. 6997-7004, a copy of which is attached hereto as Exhibit 3. The first paragraph of the Materials and Methods section of Wyatt et al. reads as follows:

> Mutant envelope glycoproteins. The HIV-1 (HXBc2 strain) envelope glycoprotein mutants used in this study were previously described (46). The Δ297-329 mutant contains a deletion spanning the V3 loop, with the sequence Gly-Ala-Gly inserted in place of the loop (62, 68). Envelope glycoproteins were expressed by transfection of ***plasmid pSVIII-env*** containing either a wild-type or mutated env gene into COS-1 cells by the DEAE-dextran technique (46). [Emphasis added.]

Thus, Wyatt et al. explicitly states that the Δ297-329 mutant described in Example 2 of the above-identified application had amino acid residues 297-329 replaced with Gly-Ala-Gly. Wyatt et al. also makes it apparent that the numbers "297-329" refer to amino acids in the protein sequence and not to base positions in the encoding nucleic acid sequence.

(23) The generation of the 1ΔV3, 7ΔV3, and 8ΔV3 mutants has been described in manuscript by Kmieciak et al., J. Immunol. 1998, 160:5676-5683 in the Materials and Methods section (a copy of Kmieciak et al. is attached hereto as Exhibit 6). The following is a summary of that method.

The HIV-1IIIB isolate was the source of the wild-type (WT) envelope (env) gene and the ΔV3 env mutant cloned in the pSC11-based vector under the control of a synthetic early/late vaccinia virus (vv) promoter. The 1ΔV3, 7ΔV3, and 8ΔV3 mutants are recombinant vv clones generated by homologous recombination of the pSC-ΔV3 plasmid using nonrecombinant vaccinia virus. They all express the Δ297-329 deletion of the env glycoprotein.

The pSC-ΔV3 env plasmid was constructed by ligation of fragments obtained by PCR amplification from the pSVIII-env plasmid. One fragment was generated by PCR with the synthetic oligonucleotide containing the *SalI* site and the CCACC Kozak's sequence in front of the ATG condon (5'-AGAGTCGACCCACCATGAGAGTGAAGGAGA-3', sense) and

with the oligonucleotide (5'-ACAGGTACCCCATAATAGACTGTGAC-3', antisense) containing the *Kpn*I site. The second fragment was derived by *Kpn*I and *BamH*I digests of the pSVIII-env plasmid, and the third fragment was generated by PCR with the synthetic oligonucleotide containing the *BamH*I site at its 5' end (5'-AACGGATCCTTAGCACTTATCTGGG-3', sense) and the antisense primer (5'-TTGCGCGGCCGCTTATAGCAAAATCCTTTCC-3') containing the TAA stop codon followed by the *Not*I site. The three fragments were ligated into the *Sal*I and *Not*I sites of the pSC-11-based vector to generate plasmid pSC-ΔV3. A similar approach was used to generate plasmid with the WT env gene (pSC-WTP) using the recombinant clone pIIIB. Plasmids pSC-ΔV3 and pSC-WTP were used to generate recombinant vv-ΔV3 (1ΔV3, 7ΔV3, 8ΔV3) and vv-WTP (WTP-2, WTP-5, and WTP-8) viruses by homologous recombination. Note, that only vv-WTP-2 and vv-7ΔV3 were used for functional studies including env-specific cytotoxic T cell responses and HIV gp120-mediated pathogenesis.

(24) The specification of the above-identified application provides support for the phrase "introducing into a vector DNA or liposome encoding an envelope..." as recited in Claim 28 at page 4, lines 14-15.

(25) The specification of the above-identified application provides support for APCs with adjuvant as recited in Claim 29 at page 4, lines 20-23.

(26) The specification of the above-identified application provides a detailed description of procedures for making the nucleic acid and cells recited in the Claims 14, 15, 19, and 21-36 of the above-identified application and a description of how to use those cells for preparing a vaccine against HIV, for inducing cellular immunity against HIV, stimulating CTL activity against HIV, and stimulating a CTL response in a patient as specified in those claims. One skilled in the field of the invention would have appreciated at the time the above-application was filed in the U.S. that the inventors thereof were in possession of the

invention as defined in the claims of the above-identified application and that those inventions could be practiced using routine experimentation.

(27) Several articles were published after the above-identified application was filed in the U.S. and demonstrate that the methods and procedures described in the above-identified application described how to make and use compositions as specified in Claims 14, 15, 19, and 21-36 of the above-identified application. Those publications are:

(a) Rowland-Jones et al., *Immunology Letters*, 1999, 00. 9-14, a copy of which is attached hereto as Exhibit 4;

(b) Kiszka et al., *Journal of Virology*, May 2002, pp. 4222-4232, a copy of which is attached hereto as Exhibit 5; and

(c) Kmieciak et al., *The Journal of Immunology*, 1998, pp. 5676-5683, copy of which is attached hereto as Exhibit 6.

(28) Rowland-Jones et al. states that eliciting a CTL response is an important goal for developing a vaccine against HIV. See the Abstract.

(29) Kiszka et al. is co-authored by the two inventors of the above-identified application, i.e., Yutaro Kaneko and Danuta Kozbor. Kiszka et al. demonstrate the "vaccines expressing the ΔV3 mutant of either HIV -1IIIb or HIV-189.6 envelope glycoproteins induced broader CD8+ T-cell activities than those elicited by the wildtype (WT) counterparts." See the Abstract.

(30) Kmieciak et al. is also co-authored by the two inventors of the above-identified application. Kmieciak et al. describe that the ΔV3 mutant described in the above-identified application increased CTL activities against conserved epitopes of the env glycoprotein. See the Abstract.

(31) The phrase "introducing into a vector DNA or liposome a nucleic acid encoding an envelope glycoprotein of HIV," as recited in Claim 28 of the above-identified

application, would not have been unclear to those skilled in the art when the above-identified application was filed in the U.S. That phrase simply means that a nucleic acid which encodes an envelope glycoprotein of HIV is introduced into a vector DNA or liposome.

(32) The meaning of the term "introducing" such a nucleic acid into a vector DNA or liposome, as recited in Claim 28 of the above-identified application, was well-known in the field of the invention at the time the above-identified application was filed in the U.S. Moreover, the specification of the above-identified application explains such a procedure in detail, and even provides specific Examples thereof.

(33) The fact that Claim 28 of the above-identified application recites vector DNA or liposome as alternatives would not have confused those skilled in the art when the above-identified application was filed in the U.S., because there is nothing confusing about the meaning of two alternatives in the claim.

(34) The meaning of the term "vector DNA" as recited in Claim 28 of the above-identified application would have been readily appreciated by those skilled in the field of the invention at the time the above-identified application was filed in the U.S. That term is defined in the specification of the above-identified application at page 9, lines 20-21.

(35) The meaning of Claim 29 of the above-identified application would have been readily appreciated by those skilled in the field of the invention at the time the above-identified application was filed in the U.S. It would have been appreciated that the adjuvant recited in Claim 29 could be the same as or different from the adjuvant specified in Claim 28.

(36) The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under

Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

(37) Further deponent saith not.

Signature

11/4/03
Date

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